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Alternative chloride transport pathways as pharmacological targets for the treatment of cystic fibrosis

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Keywords: Cystic fibrosis; Alternative chloride transport pathways; TMEM16A; SLC26A9; Anionophores

Abstract

Cystic fibrosis is a hereditary disease that originates from mutations in the epithelial chloride channel CFTR. Whereas established therapies for the treatment of cystic fibrosis target CFTR to repair its function, alternative therapeutic strategies aim for the restoration of chloride transport by the activation of other chloride transport proteins such as TMEM16A or SLC26A9 or by the application of synthetic anionophores. TMEM16A is an anion-selective channel that is activated by the binding of Ca^{2+} from the cytoplasm. Pharmacological efforts aim for the increase of its open probability at resting Ca^{2+} concentrations. SLC26 is an uncoupled chloride transporter, which shuttles chloride across the membrane by an alternate-access mechanism. Its activation requires its mobilization from intracellular stores. Finally, anionophores are small synthetic molecules that bind chloride to form lipid-soluble complexes, which shuttle the anion across the membrane. All three approaches are currently pursued and have provided promising initial results.

1. Background

The apical side of airway epithelia is covered by a thin layer of mucus, which has a protecting function and thus needs to be constantly renewed by a process called muco-ciliary clearance. This process requires the proper hydration of the epithelial surface at the air-water interface, which is ensured by the continuous transport of water across the epithelium following the secretion of salt [1]. In cystic fibrosis (CF), a hereditary disease affecting 0.03% of the Caucasian population, this process is impaired due to the loss of function of the chloride channel cystic fibrosis transmembrane regulator (CFTR) [2, 3], which in airway epithelia is predominantly expressed in ciliated cells. The impairment of proper hydration is associated with the hyper-concentration of mucus, which results in impaired clearance facilitating the emergence of infections. Different mutations of CFTR leading to cystic fibrosis either decrease its open probability or compromise its folding and trafficking to the plasma membrane [4]. Established strategies directed towards the treatment of this debilitating disease involve the restoration of the function of CFTR by pharmacological agents that either increase its open probability or improve its targeting to the membrane [5, 6]. However, the lack of protein production as a consequence of the premature termination of translation, which is a characteristic of class I mutations, and the variable therapeutic responses among patients containing different mutations of CFTR to its pharmacological rescue underscore the need for complementary approaches to tackle this condition. Recent alternative strategies thus aim at the stimulation of alternative chloride transport pathways in airway epithelia to compensate for the loss of function of CFTR. Such strategies either attempt for the activation of existing chloride transport proteins or the introduction of artificial chloride transport pathways by application of membrane-permeable carriers [5, 7]. Alternative chloride transport proteins in native respiratory epithelia encompass, amongst others, the calcium-activated chloride channel TMEM16A and the chloride transporter SLC26A9 whereas small synthetic carriers, which bind chloride and shuttle it across cellular membranes, would be applied to the tissue from the outside. These potential alternative targets for the treatment of cystic fibrosis are described in the following chapters. This review is based on two presentations at the 16th ECFS Basic Science Conference, Dubrovnik, Croatia, 27 to 30 March, 2019.

2. The calcium-activated chloride channel TMEM16A

Whereas a calcium-activated chloride conductance was observed in airway epithelia more than thirty years ago [8], the underlining molecular identity of the channel remained elusive until the discovery of TMEM16A as calcium-activated chloride channel about ten years ago [9-11]. In these epithelia, TMEM16A is predominantly expressed on the apical side of mucus-producing goblet cells [12]. The protein is activated by the elevation of the cytosolic calcium concentration as a consequence of cellular signaling processes. TMEM16A is part of the TMEM16/anoctamin family of membrane proteins that encompasses 10 paralogs in human, which either form channels for ions or lipids [13, 14]. Whereas TMEM16A and B are working as *bona fide* anion channels, their paralogs function as lipid scramblases [15]. Latter facilitate the movement of lipids between both leaflets of the bilayer thereby dissipating the lipid asymmetry and exposing the negatively charged phosphatidyl-serine to the outside, which in turn activates signaling cascades leading to blood coagulation, exocytosis and myoblast fusion [14]. Both functional branches of the family share a common molecular architecture and a conserved mechanism of calcium activation that was initially defined by the crystal structure of the lipid scramblase nhTMEM16 [16, 17] and later by the structures of murine TMEM16A determined by cryo-electron microscopy (cryo-EM) [18-20]. All TMEM16 proteins are homo-dimers of subunits that contain an N-terminal domain followed by a transmembrane unit consisting of ten membrane-spanning α-helices (Fig. 1A). The catalytic regions contained within each subunit are located at the opposite ends of the dimer and function as independent entities with respect to activation and transport [21, 22]. In TMEM16 scramblases, this region termed the 'subunit cavity' exposes a hydrophilic membrane-spanning furrow that provides a suitable pathway for lipid headgroups across the membrane [16]. In contrast, in the ion channel TMEM16A, the rearrangement of α-helices constituting the subunit cavity in nhTMEM16 have closed the access to the bilayer and instead form a protein-enclosed pore that is for most parts shielded from the membrane [18]. In the conducting state, the hour-glass shaped pore contains wide hydrophilic vestibules on both sides of the membrane narrowing to an amphiphilic neck region which conducts anions that have likely shed large parts of their hydration shell [19] (Fig. 1B). In TMEM16A, this pore does not contain specific anion binding sites and its strong anion over cation selectivity is instead a consequence of the positive electrostatic environment [18]. Activation of the protein proceeds by the binding of two Ca^{2+} ions to a site contained within the transmembrane domain of each subunit that is accessible form the intracellular side and located in the vicinity of the ion conduction pore [19] (Fig. 1C). This site consists of five negatively charged

residues that are strongly conserved within the family [16]. The location of the Ca^{2+}-binding site within the transmembrane electric field explains the observed voltage-dependence of activation with increased potency of Ca^{2+} at positive voltages [16]. The binding of Ca^{2+} to the closed channel promotes ion conduction by two distinct mechanisms: It causes a conformational change of one helix (α6), which contributes to the ion binding site and which has retracted from the pore region in the absence of Ca^{2+} [19] (Fig. 1C). The rearrangement of α6 transduces a conformational change to open a gate in the narrow pore region and it reverts the repulsive electrostatics on the intracellular pore entry conferred by the negatively charged binding site residues into a positive electrostatic environment that facilitates anion conduction [23]. Thus, in TMEM16A, Ca^{2+} directly contributes to ion conduction by a mechanism that is highly unusual for ligand-dependent ion channels. Any therapeutic strategy that is directed towards channel activation by an increase of the open probability thus also has to compensate the negative electrostatics of the vacant binding site that would hamper anion efflux, which would be best accomplished by a concomitant increase of the Ca^{2+}-potency towards nanomolar concentrations found in a resting epithelium. Noteworthy, besides activation of TMEM16A also a potential opposing strategy aiming at its inhibition has been discussed [24]. Latter proposal was motivated by the presumed role of TMEM16A in the contraction of endothelial sooth muscles and its association with mucus secretion where the proposed inhibition might prevent hypersecretion under inflammatory conditions.

3. The chloride transport protein Slc26a9

SLC26A9 is a chloride transport protein that is expressed in ciliated cells of airway epithelia. Its activity is enhanced under inflammatory conditions where it was shown to substantially contribute to epithelial chloride transport and thus to prevent mucus obstruction [25]. Whereas, upon heterologous overexpression, its localization was found to be predominantly intracellular, presumably as consequence of the interaction with WNK kinases [26], the protein was proposed to undergo functional interactions with CFTR, which potentially regulates its transport function and targeting to the plasma membrane [27-29]. SLC26A9 is a member of the large SLC26 family, which constitutes multifunctional anion transport proteins that are found in all kingdoms of life [30]. Whereas most of the ten functional paralogues in human were shown to be involved in the transport of chloride, bicarbonate, oxalate, iodide and sulfate, SLC26A5 (or Prestin) appears to

have lost its transport activity and instead works as chloride-dependent motor protein in cochlear outer hair cells [31]. Unlike the majority of mammalian family members, which function as coupled anion exchangers, SLC26A9 mediates uncoupled chloride transport and, although frequently referred to as ion channel, works as bidirectional uniporter that mediates rapid chloride transport by an alternate-access mechanism [26, 32]. Although transporting different anions by a lyotropic permeability sequence that favors larger ions with lower hydration energy, the biologically important anions bicarbonate and sulfate are not transported by SLC26A9 [32]. The general structural features of the SLC26 family were initially revealed from the crystal structure of the prokaryotic homologue SLC26Dg, working as proton-coupled fumarate symporter [33]. This structure has defined the overall organization of the family consisting of a transmembrane domain (TMD) followed by a cytoplasmic STAS domain, but it did not reveal the oligomeric organization of SLC26 transporters, which form dimers as later confirmed by a spectroscopic study of the protein [34]. The detailed oligomeric structure of SLC26A9 was uncovered by cryo-EM [32], which defined the common architecture of mammalian members of the SLC26 family (Fig. 1D). The unusual dimeric arrangement exhibits minimal interactions between the TMDs and dimerization is instead mediated by mutual contacts between the domain-swapped STAS domain, their interaction with the TMD of the neighboring subunit and the extended N-terminus of the protein (Fig. 1D). This organization differs from dimeric transporters of the SLC4 and SLC23 families, which show a similar organization of the TMD but which bury an extended interaction interface within the membrane [35]. Despite their interactions via the STAS domain, the two TMDs likely work as independent entities. They are organized by a 7+7 inverted repeat topology with two structurally related halves that are oriented with opposite orientations relative to the membrane [35]. Each TMD segregates into two interacting structural sub-domains consisting of helices of both halves, which were termed core and gate modules (Fig. 1E) The core module contains an anion-binding site located on the surface of the protein apposed to the gate module in the center of the protein (Fig 1F). This site can be reached via aqueous vestibules which, depending on the conformation of the transporter, provide access to either one or the other side of the membrane and which confer a positive electrostatic environment attracting anions on the path to the binding site [32]. In this site, the bound ion is located in a narrow pocket and interacts with polar and hydrophobic residues, which likely accounts for the observed selectivity sequence favoring larger anions (Fig. 1G). The movements of the protein are illustrated in the comparison

of two structures of SLC26A9 determined in detergent and in lipid nanodiscs, which show different conformations [32]. During transport, the gate modules and interacting STAS domains function as rigid scaffolds, whereas the mobile core module of each TMD carrying the ion binding site moves as rigid unit to change the exposure of the site to either side of the membrane, thereby crossing the transmembrane electric field in a process that was previously defined as 'elevator mechanism' [36]. Although transport appears to be constitutive and not regulated by activating ligands, the proposed functional interaction with CFTR and the signals that regulate the trafficking of SLC26A9 are still poorly understood [26, 27]. The exploitation of this protein as pharmacological target for the treatment of cystic fibrosis would thus have to improve the plasma membrane expression in absence of functional CFTR.

4. Ionophores as alternative chloride transport systems

Another potential alternative to compensate for the reduced CFTR anion transport activity are small molecules capable of facilitating the permeation of chloride and bicarbonate across epithelial membranes. These molecules, termed anionophores (for anion selective ionophores), are a recent addition to the research in cystic fibrosis [5]. Anionophores are able to coordinate anions through non-covalent interactions such as hydrogen bonds. In this way, a supramolecular complex is formed that provides a lipophilic shell for the anion thus allowing its diffusion across the membrane [37]. In stark contrast to cation-selective ionophores or pore-forming molecules, only few natural products have been identified to act as anionophores. Therefore, numerous synthetic molecules are being developed, including macrocycles such as calix[4]pyrroles and small molecules bearing convergent hydrogen bond donor groups such as amides, (thio)ureas, sulfonamides or squaramides [37]. Prodigiosin is the lead example of naturally occurring anionophores, naming a family of compounds with intriguing pharmacological properties [38]. Related molecules are marine secondary metabolites termed Tambjamines. Based on these natural compounds, synthetic analogues with anionophoric properties have been synthesized [39]. These molecules have been shown to promote anion transport into living cells in a concentration-dependent manner. Measurements of iodide influx in FTR cells expressing a halide-sensitive YFP protein allowed quantification of the anion transport activity by these compounds in relationship to the activity of CFTR. The transport efficiency was found to be pH dependent with a maximum

at slightly acidic pH values [40]. Under these conditions, the measured quenching rate is comparable to that observed for CFTR. The activity was also found to be sustained over time and no decay of quenching rate was observed for periods up to one hour. Since bicarbonate is equally important for epithelial physiology, an ideal anionophore would also be able to correct the compromised transport of this anion. Using a pH-based assay, bicarbonate transport promoted by synthetic anionophores was demonstrated in Fischer rat thyroid (FRT) cells [41]. Interestingly, the activity of synthetic anionophores has been shown to be additive to the pharmacological rescue of CFTR activity [41]. Thus, in FRT cells expressing G551D-CFTR, rescue of the mutated-CFTR activity by ivacaftor was additive to that driven by anionophores. Similar results were observed when the F508del-CFTR activity was rescued by the corrector lumacaftor.

The use of cholic acid as scaffold provided a different series of highly active anionophores [42]. This steroidal framework allows the placing of up to three (thio)-urea groups in an axial, convergent disposition, thereby creating a polar pocket suitable to bind spherical anions. These anionophores, termed cholapods, have been shown to promote anion transport in FRT cells and their activity was further confirmed by electrophysiological studies in epithelia [43]. Using the Ussing chamber technique anionophore mediated Cl^- currents in the apical membrane of FRT epithelia were recorded.

An encouraging result involving the use amphotericin B was recently reported [44]. Amphotericin B is a channel-forming molecule capable of facilitating unselective ion permeation in membranes. In this study, Amphotericin B restored airway surface liquid pH, viscosity and antibacterial activity in cultured airway epithelia derived from genetically diverse humans with CF. Using a clinically approved formulation of this compound (termed AmBisome) the restoration of airway surface liquid pH *in vivo* was demonstrated in a porcine model of CF. The success of this approach offers further evidence for the potential of small molecules acting as CFTR surrogates for correcting defective anion transport. Although the toxicity resulting from an exogenous transport mechanism and the selective targeting of small molecule anionophores to the apical membranes remain important issues that require further studies, current data indicate that the use of anionophores provide a valuable strategy in the treatment of cystic fibrosis.

5. Summary

In this review, we have described three transport systems that facilitate the flow of chloride ions across the apical membrane of airway epithelia, which could compensate for the loss of activity of the CFTR channel. These three systems operate by different mechanisms with TMEM16A functioning as Ca^{2+}-gated ion channel where the ligand also plays an important role in shaping the conductance properties, SCL26A9 as uncoupled bidirectional transporter, which potentially interacts with CFTR and anionophores acting as mobile carriers, which bind chloride to permit its diffusion across the membrane (Figure 2C). The restoration of chloride transport could either proceed by the activation of the two native transport proteins or the introduction of ionophores to the apical side of the epithelia. In this respect, the exploitation of either transport system presents a promising alternative therapeutic strategy for the treatment of cystic fibrosis. Whereas two approaches, namely the activation of the ion channel TMEM16A by potentiators [45] and the use of ionophores [44] are currently pursued and have provided promising initial results, investigations on the activation of SLC26A9 are still at an early stage [28].

Conflict of interests

The authors have no conflicts of interest to declare.

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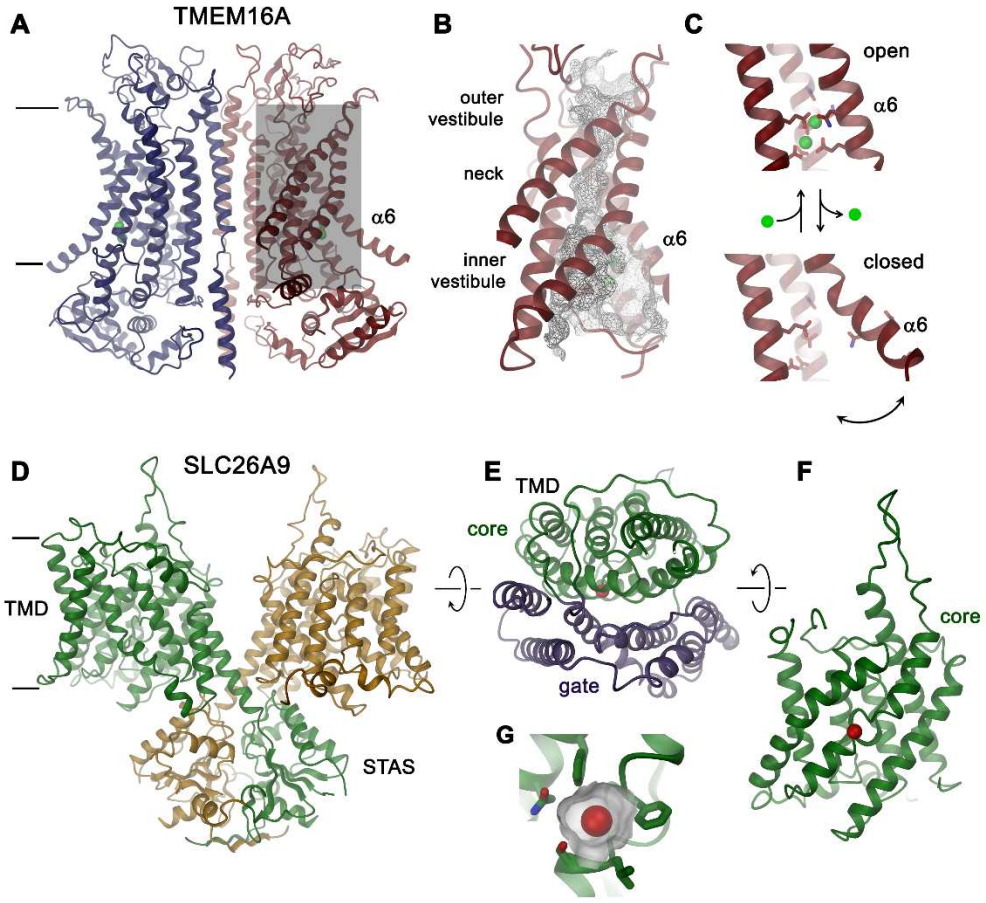

Fig. 1. Anion transport proteins in airway epithelia. A. Ribbon representation of the dimeric Ca^{2+}-activated chloride channel TMEM16A viewed from within the membrane. Membrane boundaries are indicated. Region containing the ion conduction pore in one of the subunits is highlighted as grey box. B. Closeup of the anion permeation pore. The molecular surface of the pore is shown as grey mesh. C. Conformational changes upon Ca^{2+}-binding to a site contained within the transmembrane domain. Top, Ca^{2+}-bound open conformation, bottom, Ca^{2+}-free closed conformation, with movements of α6 indicated by arrow. A-C, bound Ca^{2+} ions are shown as green spheres, the α-helix involved in gating (α6) is labeled. D. Ribbon representation of the dimeric chloride transporter SLC26A9 viewed from within the membrane. Transmembrane domain (TMD) and cytoplasmic STAS domain (STAS) are labeled and membrane boundaries indicated. E. Single TMD viewed from the extracellular side with core and gate modules labeled and shown in unique colors. F, Core module viewed from within the membrane. G. Closeup of the anion binding pocket with side-chains of surrounding residues displayed as sticks. Molecular surface of the binding pocket is shown in grey. E-G, bound Cl^- ion is shown as red sphere.

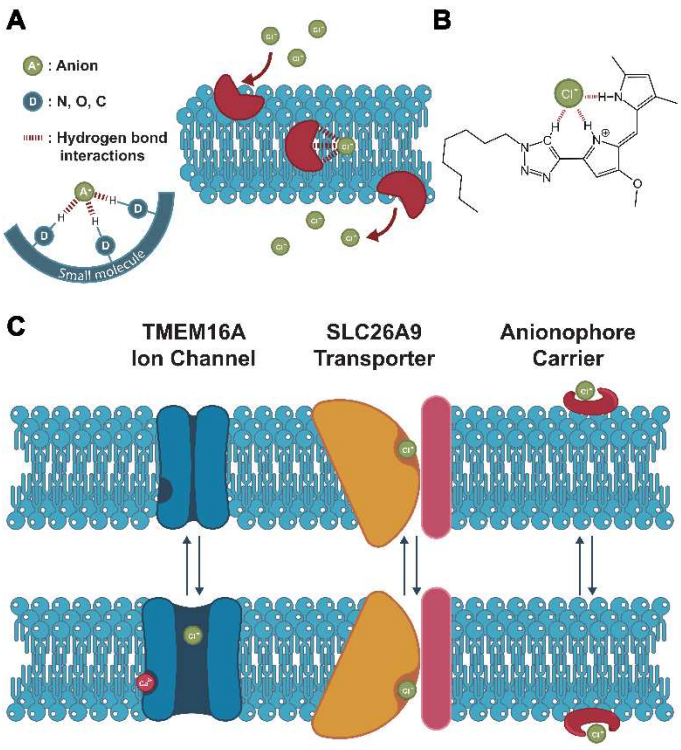

Fig. 2. A. schematic view of the complexation of Cl^- by an anionophore (left) and its transport across the membrane (right). B. Chemical structure of an anionophore derived from the natural compound Prodigiosin. C. Schematic summary of transport mechanisms. Left, the Ca^{2+}-activated ion channel TMEM16A; Center, the transporter SLC26A9; Right, a synthetic anion-selective carrier. A-C, Cl^- and Ca^{2+} are depicted as green and red spheres respectively.